



Construction and characterization of heterodimeric soluble quinoprotein glucose dehydrogenase

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Abstract

In order to study in greater detail the subunit interaction of the homodimeric soluble quinoprotein glucose dehydrogenase (PQQGDH-B), we developed an effective method of creating heterodimeric PQQGDH-B. Two different homodimers are combined, one of which has a polyarginine tail (Arg-tail), and subjected to a protein dissociation/redimerization procedure. Separation of the mixture by cation exchange chromatography results in three peaks showing GDH activity, eluting at 133, 231 and 273 mM NaCl concentration. These peaks were determined to correspond to the Arg-tailless homodimer, heterodimer, and Arg-tailed homodimer, respectively. To test this approach, we constructed and characterized heterodimeric PQQGDH-B composed of native (wild-type) and inactive mutant (His168Gln) subunits. The heterodimeric wild-type-His168Gln showed slightly decreased GDH activity and almost identical substrate specificity profile to the wild-type enzyme. Moreover, the Hill coefficient of the heterodimer was calculated as 1.13, indicating positive cooperativity.

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1. Introduction

Soluble quinoprotein glucose dehydrogenase (PQQGDH-B), because of its high catalytic efficiency (k_{cat}/K_m) and independence of dissolved oxygen, is a promising

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alternative to glucose oxidase (GOD) for blood glucose monitoring [1]. Unfortunately, PQQGDH-B also possesses two inferior properties to GOD, substrate specificity and thermal stability. We have therefore been pursuing the improvement of enzymatic properties of PQQGDH-B through protein engineering approaches [2–8].

PQQGDH-B is a dimeric enzyme made up of identical 50-kDa subunits with W6-bladed β -propeller structures [9–11]. Although the active sites are located in the center of each propeller structure, the dimeric status is essential for GDH activity. We have reported several mutant PQQGDH-Bs with altered enzymatic properties [2,4–8]. Considering the enzyme's quaternary structure, the introduction of a single amino acid substitution would appear at an identical position in each subunit of the homodimeric mutant enzyme. Therefore, the observed altered enzymatic properties result from the sum of two mutations per homodimeric enzyme. The mechanism by which dimer association results in the expression of enzyme activity must first be elucidated for the further progress in engineering of this industrially significant enzyme.

In this paper, we report the development of a method to create heterodimeric PQQGDH-B, in which each subunit harbors different mutations, in order to study the dimeric status of PQQGDH-B in greater detail. Wild-type enzyme with a C-terminal tail of four consecutive Arg residues was mixed with mutant PQQGDH-B. Heterodimeric enzyme was readily purified by cation exchange chromatography after heat dissociation and redimerization. As a representative mutation, we chose His168Gln, which is located at the active site and had been shown to result in a drastic decrease in the enzymatic activity [7].

2. Materials and methods

2.1. Strains and plasmids

All PQQGDH-B genes were inserted into the multicloning site of the expression vector pTrec99A (Pharmacia, Sweden) and expressed in *Escherichia coli* PP2418, in which the membrane-bound PQQGDH structural gene was disrupted by insertion mutagenesis [13].

2.2. Genetic manipulation

Based on our previous study [14], we introduce a tail of four consecutive Arg residues [15] at the C-terminus of wild-type PQQGDH-B by using the following set of oligonucleotide primers: 5'-GGCCATGGATAAACATTTATTGGCTAAAATTGCTT-TAT-3' (Forward) and 5'-CCAAGCTTTAGCGCCTTCGACGCTTAGCCTTATAGGT-GAACTTAATGAGAG-3' (Reverse). Amplification by PCR was performed using the wild-type gene as the template. The amplified fragment (1.5 kb) was digested by *Nco*I and *Hind*III and inserted into pTrec99A. The nucleotide sequence was confirmed by 310 Genetic Analyzer (Applied Biosystems CA, US). The resulting expression vector was named Wild-type+Arg4.

2.3. Preparation of heterodimeric enzyme

Wild-type+Arg4 and the inactive mutant His168Gln were prepared as previously reported [2,4]. The two enzymes (200 μ g each) were combined in 10 mM potassium phosphate buffer (pH 7.0) to form the heterodimeric enzyme as outlined in Fig. 1. The mixture was incubated at 50 °C of 5 min to dissociate the quaternary structure of each enzyme. The sample was then incubated at 4 °C in the presence of 1 μ M PQQ and 1 mM CaCl_2 for 5 min to allow redimerization. The sample was then immediately applied onto a cation-exchange column (Resource S, 1 ml; Amersham Biosciences Corp. NJ, US) and eluted with a linear NaCl gradient (70–350 mM for 12 min) in 10 mM potassium phosphate buffer (pH 7.0) in 3 ml/min. The resulting fractions were dialyzed separately in 10 mM MOPS–NaOH (pH7.0) and assayed for their enzyme activity. The

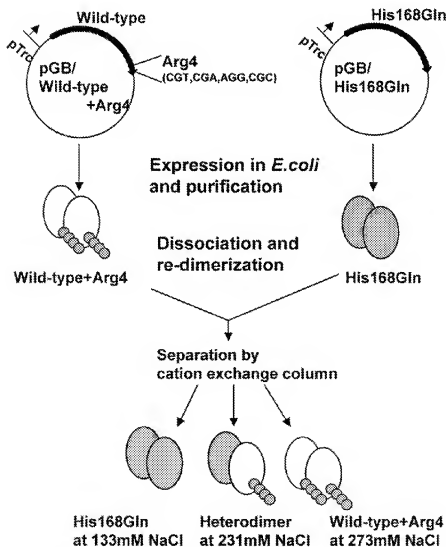


Fig. 1. Schematic depiction of the isolation of heterodimeric PQQGDH-B from parental homodimeric PQQGDH-Bs.

enzyme sample thus prepared was stored in 10 mM MOPS–NaOH (pH 7.0) containing 1 μ M PQQ and 1 mM CaCl_2 .

2.4. Enzyme assay

GDH activity was measured using 0.6 mM phenazine methosulfate (PMS) and 0.06 mM 2,6-dichlorophenolindophenol (DCIP) after incubation in 10 mM MOPS–NaOH (pH 7.0) for 30 min in the presence of 1 μ M PQQ and 1 mM CaCl_2 . Each activity at several concentrations of glucose was calculated by monitoring the decrease in absorbance of DCIP at 600 nm. The evaluation of substrate specificity profiles was performed using allose, 3-*O*-methyl-glucose, galactose, lactose and maltose at several concentrations.

2.5. SDS-PAGE

SDS-PAGE was carried out on 8–25% gradient gels with Phast system (Amersham Biosciences Corp. NJ, US) according to the manufacturer's instructions.

3. Results and discussion

3.1. Construction of heterodimeric PQQGDH-B

We recently reported on the fusing of an Arg tail to PQQGDH-B to achieve more efficient protein separation [14]. The addition of an Arg tail on PQQGDH-B resulted in a considerable delay in elution during cation exchange chromatography compared with wild-type enzyme, while showing similar GDH activity (4512 U mg^{-1}) to wild-type (4610 U mg^{-1}) and a similar substrate specificity profile. These observations indicated that the kinetic properties were unaffected by the presence of the additional positive charge at the C-terminus.

We investigated the possibility of applying the Arg tail for isolating heterodimeric PQQGDH-B, with one wild-type subunit and one subunit from the inactive His168Gln mutant. Purified wild-type+Arg4 and His168Gln were combined and subjected to heat dissociation and redimerization procedures. Separation of the mixture by cation exchange chromatography resulted in three main peaks, eluting at 133, 231, and 273 mM NaCl concentrations, respectively (Fig. 2A). Applying wild-type+Arg4 and His168Gln separately resulted in single peaks, corresponding to peaks 3 and 1, respectively. Furthermore, subjecting a mixture of the two proteins to cation exchange chromatography, without the dissociation and redimerization procedures, peak 4 and peak 5 are observed (Fig. 2B). These are same positions as peaks 1 and 3 (Fig. 2A and B). A peak corresponding to peak 2 was not detected. We therefore concluded that peaks 1 and 3 contain His168Gln and wild-type+Arg4, while peak 2 contains a heterodimer resulting from the dimerization of wild-type+Arg4 and His168Gln. These elution properties are consistent with the assumed structure of heterodimeric PQQGDH-B that harbors one subunit with an Arg tail. Moreover, when we adjusted the molar ratios of Arg-tailed

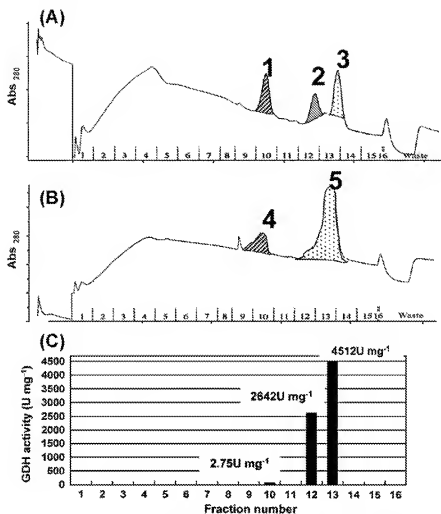


Fig. 2. Results of cation exchange chromatography of PQQGDH-Bs. (A) homodimeric and heterodimeric PQQGDH-Bs. (B) Chromatogram with heterodimerization procedure, chromatogram without heterodimerization procedure. (C) Relationship between fraction and GDH activity.

PQQGDH-B to native one, the difference in elution behavior is observed. This is also supported that peak 2 contains a heterodimeric PQQGDH-B (data not shown).

As shown in Fig. 2C, all three peaks showed GDH activity. As expected, peak 1 (His168Gln) showed very weak activity (2.75 U mg^{-1}) and peak 3 showed very high activity (4512 U mg^{-1}). The heterodimeric enzyme retained considerable activity (2642 U mg^{-1}). Considering our results, we identified peak 2 as the elution sample of heterodimeric PQQGDH-B.

Several groups achieved separation of heterodimers (hybrid dimers) from both parental homodimers using N- or C-terminal tags of six histidines (His-tag), FLAG, polyarginine (Arg-tail), or polyglutamate (Glu-tail) [16–22]. We had previously found that Arg-tailed PQQGDH-B showed increased affinity to a cation exchange column and could be effectively separated from wild-type enzyme. We have now successfully extended the use of the polyarginine tail to the isolation of heterodimeric PQQGDH-B.

Polyarginine tails have been used for increasing the isoelectric points of proteins and have been especially effective in aiding cation exchange chromatography [15]. Addition of an Arg tail onto glutathione reductase, an acidic enzyme, resulted in the requirement of three-step purification by ion-exchange chromatography (pH 6.6), chromatofocusing (pH 4–5), and gel filtration (pH 7.5) [19]. In contrast, the presence of an Arg tail on one or both subunits of PQQGDH-B allowed for the complete separation by cation exchange chromatography using the same buffer. We now have a simple, efficient, and inexpensive method of preparing PQQGDH-B.

3.2. The properties of heterodimeric PQQGDH-B

The wild-type-His168Gln heterodimeric PQQGDH-B, composed of native and inactive mutant subunits, showed significant GDH activity (2642 U mg^{-1}). Although one subunit was essentially inactive, the production of a dimeric structure lead to the formation of the active site and hence the expression of GDH activity. The K_m value of wild-type-His168Gln for glucose is 22.0 mM , which is almost identical to that of wild-type+Arg4 (23.0 mM). The heterodimer also showed almost identical K_m values as wild type toward other substrates (allose: 36.0 and 34.0 mM , 3-*O*-methyl-glucose: 22.0 and 23.0 mM , galactose: 9.0 and 8.0 mM , lactose: 20.0 and 20.0 mM , and maltose: 13.0 and 14.0 mM , respectively). Judging from the ratios of V_{\max}/K_m values, the substrate specificity profile of wild-type-His168Gln is almost indistinguishable from that of wild-type (Table 1). Because GDH activity of each active site is different, we displayed activity as V_{\max} instead of k_{cat} .

The k_{cat} value of the wild-type subunit within the heterodimer, which is the only one of the two subunits that shows activity, could be calculated as 4424 s^{-1} . Because this is higher than the homodimeric wild-type k_{cat} value of 3778 s^{-1} , a cancellation of cooperativity between each active site is considered. From the enzymatic reaction curves, Hill coefficients for the heterodimeric wild-type-His168Gln and homodimeric wild type (wild-type+Arg4) were calculated to be 1.13 and 0.80 , respectively. The cooperativity effect leads to a decrease in GDH activity per subunit of homodimeric enzyme compared with that of the wild-type subunit in the heterodimer. This observation is consistent with

Table 1
Kinetic parameters of PQQGDH-Bs for various substrates

	Heterodimer wild-type-His168Gln			Wild-type+Arg4		
	K_m (mM)	V_{\max} (U mg^{-1})	V_{\max}/K_m ($\text{U mg}^{-1} \text{ mM}^{-1}$)	K_m (mM)	V_{\max} (U mg^{-1})	V_{\max}/K_m ($\text{U mg}^{-1} \text{ mM}^{-1}$)
Glucose	22.0	2642	120 (100%)	23.0	4512	196 (100%)
Allose	36.0	2260	63 (52%)	34.0	3404	100 (52%)
3- <i>O</i> -methyl- glucose	22.0	2689	122 (101%)	23.0	4071	177 (93%)
Galactose	9.0	324	36 (30%)	8.0	471	59 (30%)
Lactose	20.0	1519	76 (63%)	20.0	2563	128 (65%)
Maltose	13.0	1507	116 (96%)	14.0	2463	176 (91%)

Substrate specificity of each enzyme is shown by comparing catalytic efficiency (V_{\max}/K_m) toward each substrate. The catalytic efficiency of each enzyme toward glucose is set as 100%.

the previously reported negative cooperativity effect of PQQGDH-B [12]. Considering that the wild-type subunit of the heterodimer showed high catalytic activity, the possibility of a cooperative effect by the electron mediator is discarded. Therefore, we propose that a negative cooperativity effect occurs between each active site of PQQGDH-B.

Homodimeric PQQGDH-B mutants have only permitted investigation of the effects of modifying identical residues on each subunit. We had been unable to introduce different mutations at any position on the enzyme subunits. We have now succeeded in the isolation of heterodimeric PQQGDH-B altered in the active site region. This is allowing us to answer some of the questions left unanswered in previous studies of mutations in homodimeric PQQGDH-B.

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